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**Kozumbo - Novel Genetic Tools**

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CARNEGIE INSTITUTION OF WASHINGTON**

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Final Report**

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# **Novel genetic tools to accelerate our understanding of photosynthesis and lipid accumulation**

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**Final Report for AFOSR YIP Grant Number FA9550-11-1-0060**

**AFOSR/RSL Bioenergy Program**

**Program manager: Dr. Patrick Bradshaw**

## I. Summary

Green algae are a promising source of biofuel, but the genetic tools for studying them are very limited. In this AFOSR YIP project, a transformative genetic tool, ChlaMmeSeq, was developed to increase the throughput of genetic screens in the green alga *Chlamydomonas* by ~1,000-fold. ChlaMmeSeq can be applied to pools of tens of thousands of mutants to simultaneously (i) determine the sites of insertion of an exogenous DNA fragment into the genomes and (ii) track the growth rates of individual mutants. ChlaMmeSeq was used to reveal the insertion sites in tens of thousands of insertion mutants, an unprecedented scale for green algae. The data revealed insights critical for future screens and for improving expression of foreign genes in *Chlamydomonas*: (i) Transforming DNA is cleaved at specific sequences, likely by a sequence-specific endonuclease; (ii) In a fraction of the mutants, genomic DNA, likely from lysed cells, is inserted between the transforming DNA and the flanking genomic DNA. Furthermore, ChlaMmeSeq was used to quantify the growth rates of ~10,000 mutants in a pool, under photosynthetic and non-photosynthetic growth. ~80 mutants with photosynthetic growth defects were identified and are the subject of current study. Finally, a fluorescence-activated cell sorting method compatible with ChlaMmeSeq was developed to enable screens for mutants with high lipid content. The new method allows isolation of mutants with high lipid contents with unprecedented speed (5 weeks), at unprecedented scale (tens of thousands of mutants), with unprecedented accuracy (50% of isolated mutants are confirmed to have a high lipid content). High-lipid mutants were isolated and are the subject of present study.

## II. Objectives

Biofuels derived from algal lipids have the potential to enable the U.S. Air Force to power its aircraft at a predictable cost basis, in the face of declining global oil reserves and an uncertain geopolitical future. However, existing algal lipid production schemes are expensive and inefficient, in large part due to our limited knowledge of algal lipid metabolism and photosynthesis. Advances in our basic understanding of these processes will facilitate genetic engineering of algae to improve lipid yields. Currently, one of the greatest roadblocks in the study of algal photosynthesis and lipid metabolism is the slow pace of discovery of the genetic components of these pathways.

This ambitious Air Force Young Investigator Program (YIP) grant FA9550-11-1-0060 aimed to develop a transformative screening tool for the model green alga *Chlamydomonas reinhardtii*. The proposed tool was designed to allow simultaneous measurement of growth rates of thousands of individual strains in a pooled culture, through the use of a unique DNA barcode in each strain's genome. Once this tool was established, we aimed to apply it to identify new genes with roles in photosynthesis and lipid accumulation in *Chlamydomonas reinhardtii*.

Our research plan had the following three aims:

### **Aim 1: Develop a transformative genetic screening tool for *Chlamydomonas*.**

We aimed to develop the ChlaMmeSeq tool, which would allow simultaneous genotyping of thousands of green algal strains in a pooled culture and the measurement of their growth rates.

**Aim 2: Comprehensively identify genes with roles in photosynthesis.**

We aimed to use ChlaMmeSeq to identify candidate genes with roles in photosynthesis. ChlaMmeSeq would be used to measure growth rates for thousands of mutants under conditions requiring or not requiring photosynthesis.

**Aim 3: Comprehensively identify mutations that perturb cellular lipid content and accumulation.**

We aimed to use the tool developed in Aim 1 to identify mutants which cause increased or decreased lipid accumulation as measured by staining with a lipophilic dye, Nile Red.

### III. Status of Effort

In Aim 1, we have successfully developed the ChlaMmeSeq tool and applied it to gain insights into the mechanisms of insertional mutagenesis in *Chlamydomonas*. Our work was reported in a publication in the top primary research journal in plant biology, The Plant Cell (Zhang *et al.*, 2014). The tool has subsequently been used in another project, where a genome-wide collection of *Chlamydomonas* mutants is being generated (supported by a grant from the National Science Foundation). A manuscript describing the first stage of this latter work is in preparation.

In Aim 2, we successfully measured the growth rates of thousands of mutants in a pooled culture and identified mutants with a specific defect under photosynthetic conditions. The identified mutants are currently being characterized and a manuscript describing this work is in preparation.

In Aim 3, we successfully developed a strategy for isolating large numbers of *Chlamydomonas* mutants with increased lipid content. A manuscript in which we demonstrate this strategy has received good reviews at a top research journal and is currently in revision. Using this strategy, we have isolated hundreds of strains enriched for high- and low- lipid - accumulating mutants. We are presently scaling up the screen to a genome-saturating scale.

### IV. Accomplishments/New Findings

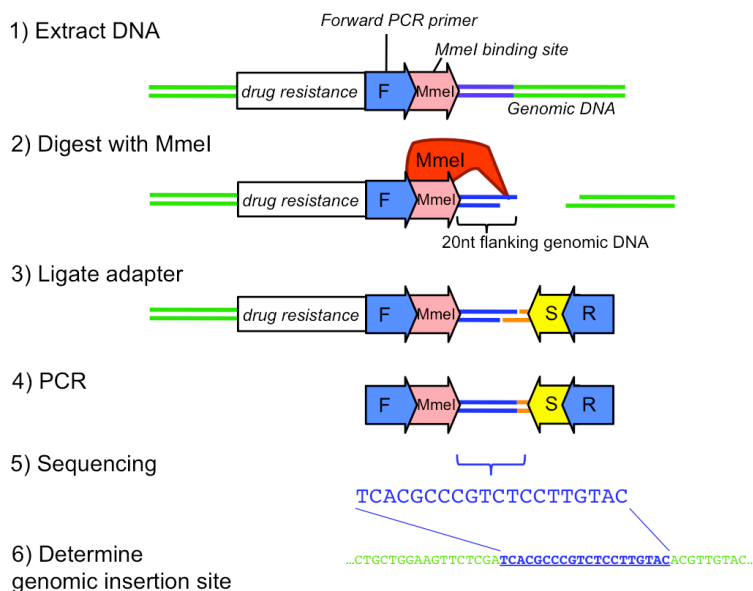
**Aim 1: Develop a transformative genetic screening tool for *Chlamydomonas*.**

We have successfully developed and demonstrated ChlaMmeSeq, a cutting-edge genotyping tool for the model green alga *Chlamydomonas reinhardtii* (Figure 1). This tool enables the identification of insertion sites in tens of thousands of individual strains in a pooled culture (Figure 2).

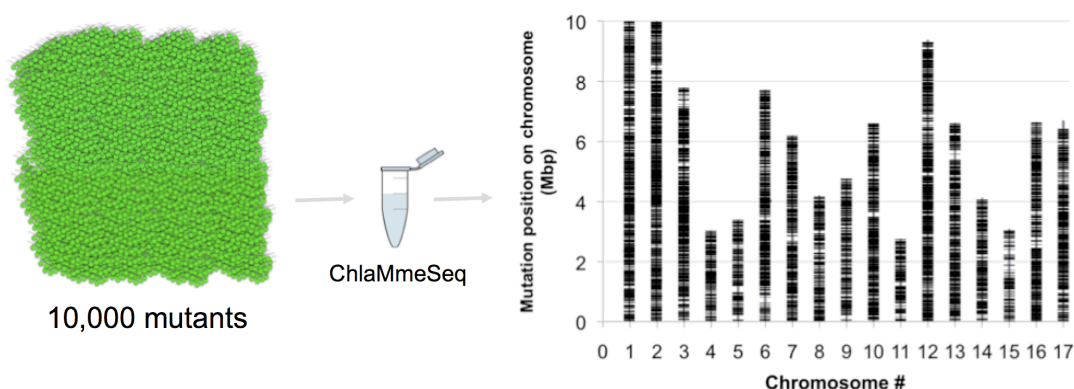
ChlaMmeSeq was used to reveal the insertion sites in tens of thousands of insertion mutants, an unprecedented scale for green algae. The data revealed that insertions are distributed throughout the genome in a manner indistinguishable from random (Figure 3). This implies that mutants in nearly all genes of this alga can be obtained efficiently by electroporation of a DNA cassette.

The data further indicate that a sequence-specific endonuclease cleaves the transforming DNA at CATG sites and variants of this sequence. This activity suggests a possible explanation for why researchers have had difficulty in expressing foreign genes in *Chlamydomonas*, and suggests that expression could be improved by removing CATG sequences and variants thereof from expression constructs.

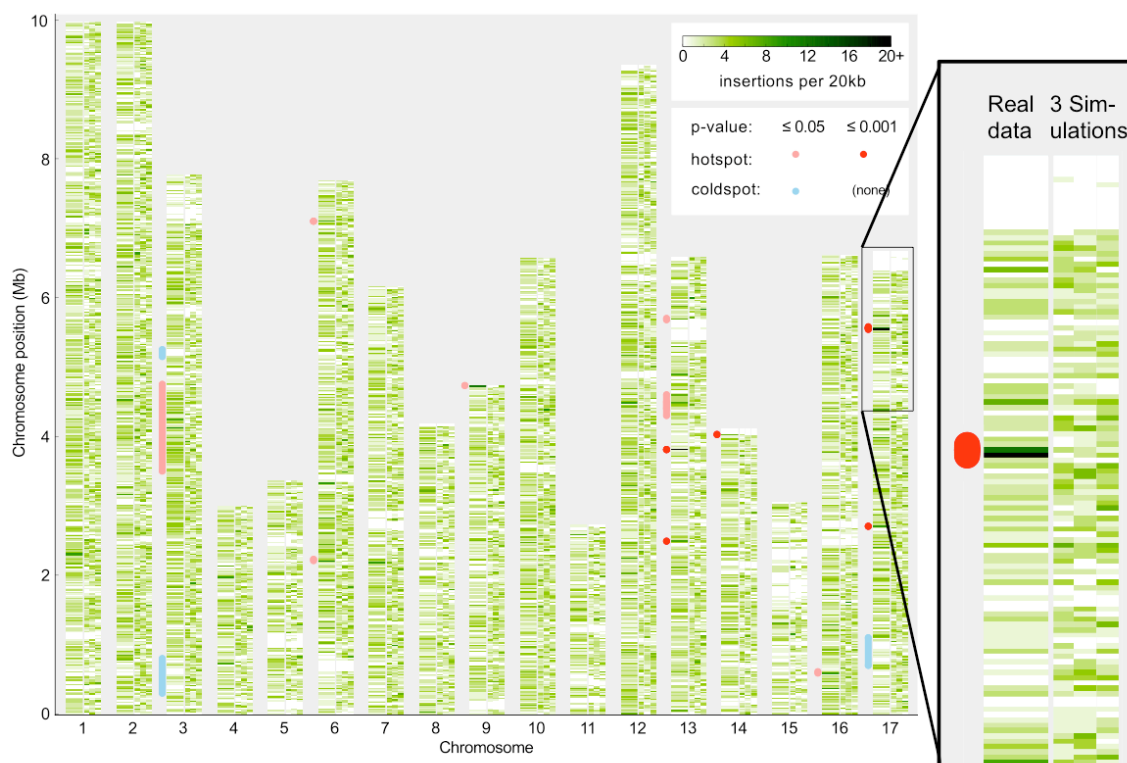
These findings are published in the top primary research journal in plant biology, *The Plant Cell* (Zhang *et al.*, 2014).



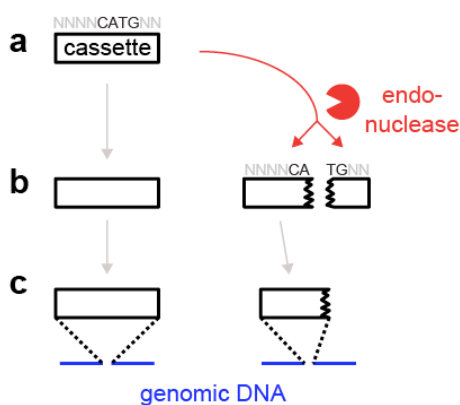
**Figure 1: ChlaMmeSeq uses *MmeI* sequence to reveal the DNA sequence flanking an insertion.** DNA from mutant(s) of interest is extracted and digested with *MmeI*. An adapter is ligated, and Polymerase Chain Reaction is performed, followed by sequencing of the region of interest using Sanger or Illumina sequencing.



**Figure 2: ChlaMmeSeq allows simultaneous mapping of thousands of insertion sites in pools of green algal mutants.** Thousands of mutants are pooled into one culture, the ChlaMmeSeq protocol is applied, and insertion sites are identified by next-generation sequencing. The graph on the right indicates actual insertion sites from a pool of 500 mutants; data from a pool of ~60,000 mutants is published (Zhang *et al.*, 2014).



**Figure 3: There are few statistically significant hot and coldspots.** For each chromosome, 4 columns are shown: the first, wider green column depicts the observed insertion density; the next three columns show insertion densities for three simulated datasets. The blue and red marks on the left of each chromosome indicate statistically significant hotspot and coldspot locations. The area containing the most significant hotspot is enlarged in the box on the right.



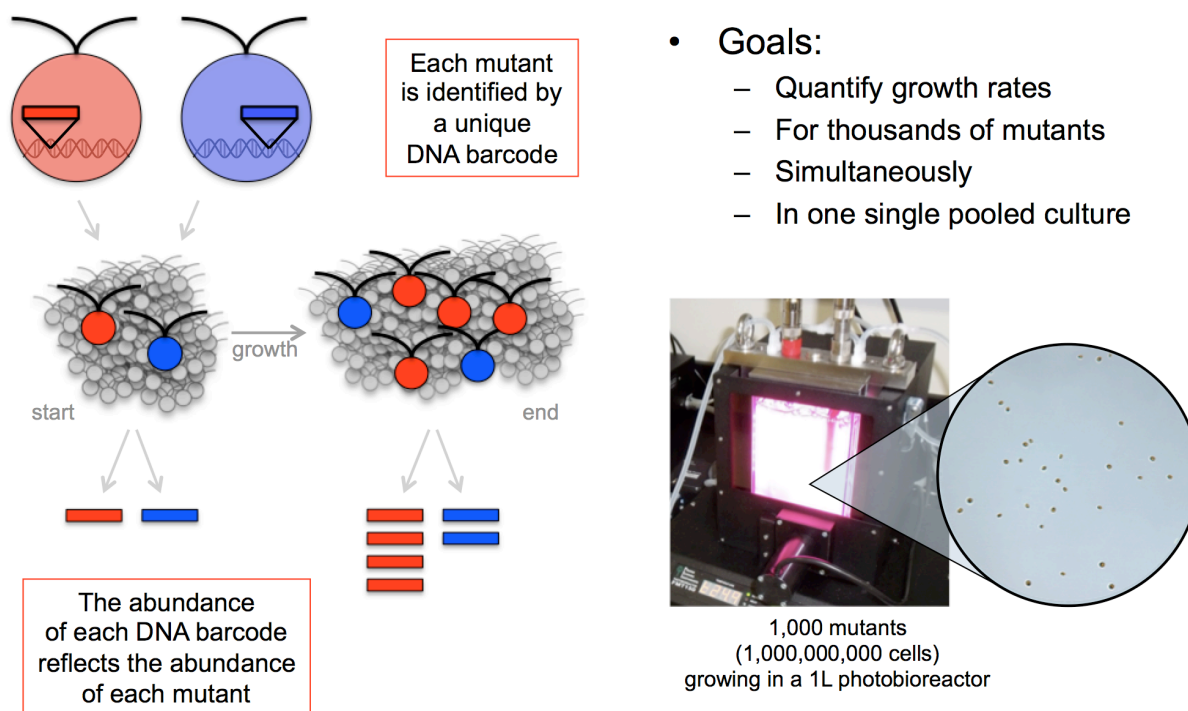
**Figure 4: Our data are consistent with a model where an endonuclease cleaves the transforming DNA, which is ligated into the genome at the site of a double-stranded break.** (a) Before transformation, the extracellular medium contains the transformation cassette. (b) During transformation, some of the cassette DNA molecules are cleaved by site-specific endonucleases at CATG sites. (c) After electroporation, intact and fragmented DNA cassettes are ligated into a double-stranded break in the genome.

## Aim 2: Comprehensively identify genes with roles in photosynthesis.

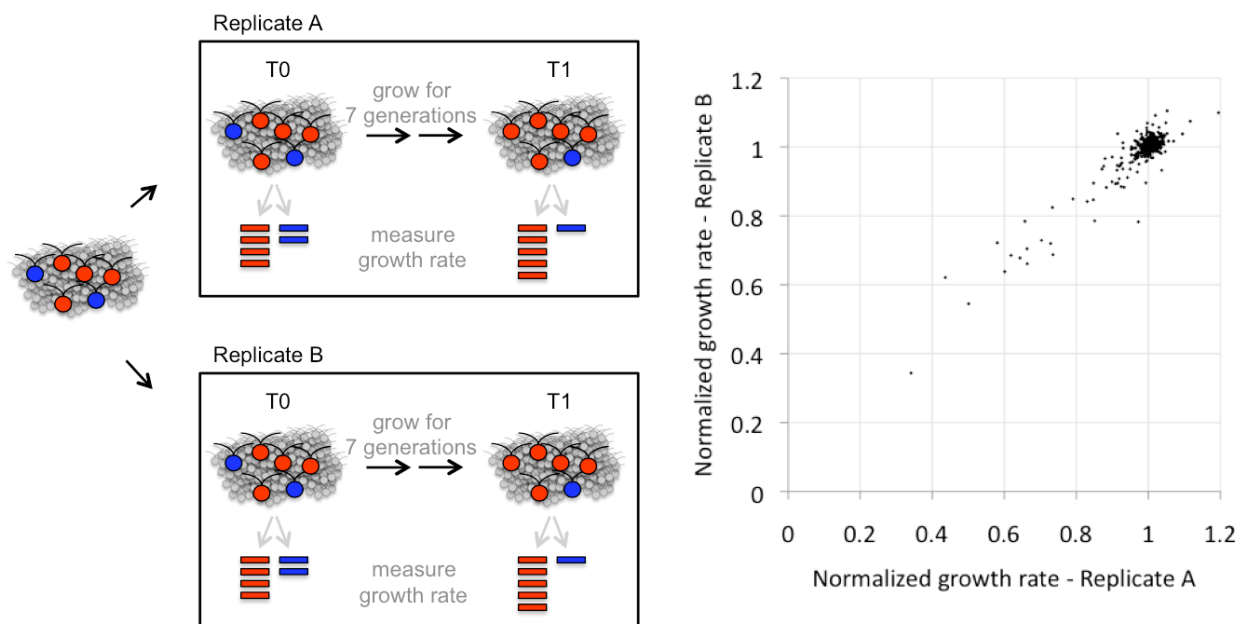
In addition to identifying insertion sites in mutants, the ChlaMmeSeq tool was designed to allow tracking of the growth rates of individual mutants in a pooled culture (Figure 4).

We demonstrated extremely accurate measurement of growth rates using ChlaMmeSeq in a pool of 500 mutants. A growth defect as small as 10% can be reproducibly identified (Figure 5). To date, ChlaMmeSeq was demonstrated to track growth rates of up to ~4,000 mutants in a pool. The next generation of this tool is under development, and preliminary data suggest that it will allow the tracking of growth rates for ~100,000 mutants in a pool.

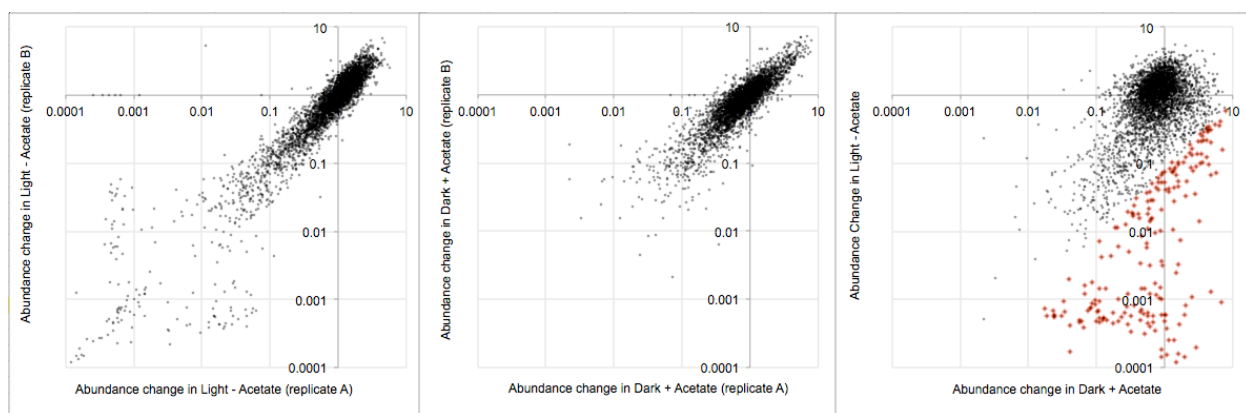
ChlaMmeSeq was used to perform a screen to identify mutants with defects in photosynthesis (Figure 6). 4,000 strains were grown in duplicate under conditions requiring photosynthesis; and under conditions not requiring photosynthesis. 195 strains were identified that exhibited significant growth defects specifically under conditions requiring photosynthesis. These strains included several dozen mutants in novel genes, which are being followed up on. A manuscript is in preparation describing our findings.



**Figure 4: Our tools allow simultaneous tracking of growth rates of thousands of mutants grown in one culture.** Thousands of mutants are pooled into one culture, which is grown over 5-7 generations. The abundance of each mutant can be determined at the starting and ending time point by quantifying the abundance of each mutant's unique barcode carried in its genomic DNA.



**Figure 5: Our growth rate measurements are highly quantitative.** The growth rates of mutants in a pool of 500 mutants were quantified in two biological replicates. The measured growth rates in the two replicates are plotted on the right.



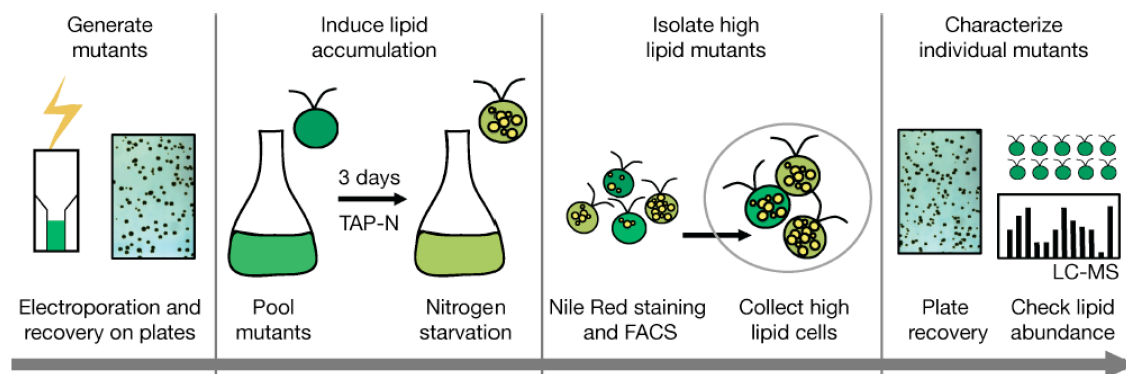
**Figure 6: Pooled growth screening identifies candidate photosynthesis-deficient mutants.** The growth of mutants in a pool of 4,000 mutants was quantified in two biological replicates under a condition requiring photosynthesis (Light - Acetate; left panel), and a condition where photosynthesis is not required (Dark + Acetate; middle panel). 195 mutants with significantly slower growth in the condition requiring photosynthesis were identified as candidate photosynthesis-deficient mutants (right panel; highlighted in red).

### Aim 3: Comprehensively identify mutations that perturb cellular lipid content and accumulation.

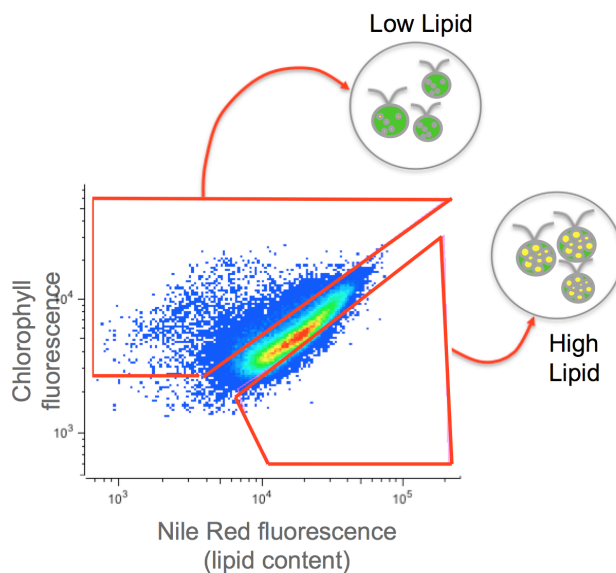
A procedure was developed for isolating mutants with unusually high or low lipid content by fluorescence-activated cell sorting (Figure 7). The procedure is remarkably rapid and produces a much greater enrichment in desired mutants than any previously existing approach.

Several innovations were implemented to improve the isolation of mutants with perturbed lipids. A detergent was added to improve dye penetration into the cells. Each cell's chlorophyll fluorescence was used as an internal control to normalize its lipid signal (Figure 8). The resulting greater sensitivity allows impressive enrichments, to a point where 50% of the isolated mutants in the high-lipid gate have increased lipid content as verified by gas chromatography-flame ionization detection and liquid chromatography-mass spectrometry.

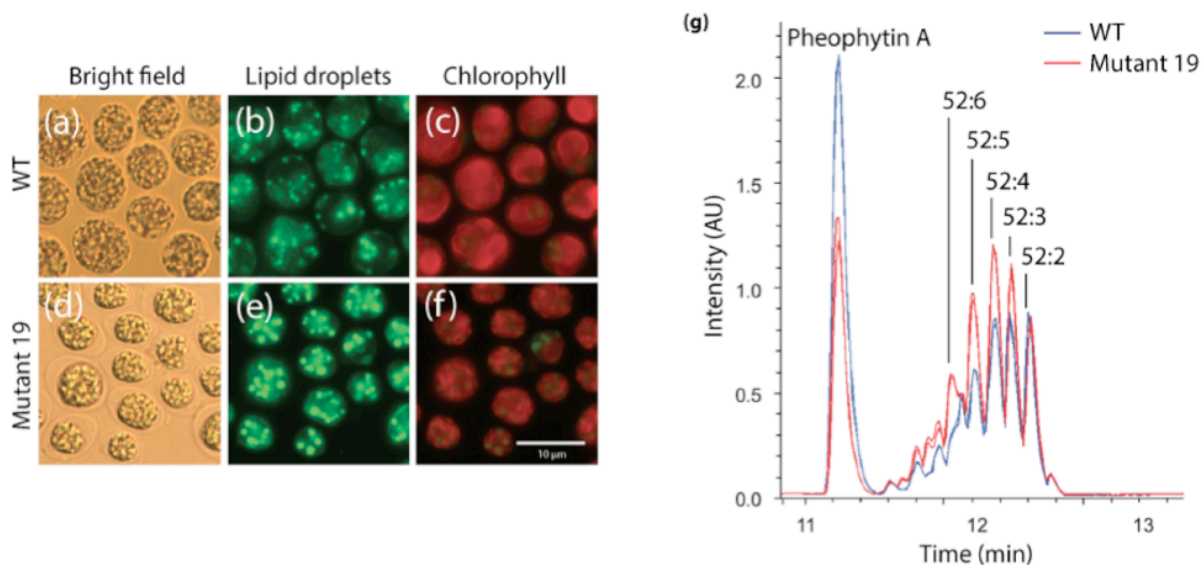
We are currently following up on a number of mutants with perturbed lipid content, with the goal of understanding key steps in lipid metabolism (such as export of fatty acids from the chloroplast) and regulation (such as identifying the master "lipid trigger" that regulates lipid accumulation). An example mutant with perturbed lipid content is shown in Figure 9.



**Figure 7: We developed a procedure to isolate mutants with perturbed lipid content.** The entire procedure takes only 5 weeks and yields a ~20x enrichment in high-lipid mutants.



**Figure 8: High and low lipid mutants are identified by differences in chlorophyll fluorescence and Nile Red fluorescence.** The sorting gates used for isolating low- or high-lipid mutants are indicated.



**Figure 9: An example mutant isolated by our strategy shows increased lipid content by microscopy and mass spectrometry.** (a-f) Microscopy analysis of Mutant 19 shows that: (a,d) it has perturbed morphology; (b,e) it has bigger lipid droplets; (c,f) it has lower chlorophyll than wild-type (WT). (g) Liquid chromatography- mass spectrometry was performed on wild-type and Mutant 19 to analyze triacylglycerol species in the strains. The data confirms that Mutant 19 has lower chlorophyll (as indicated by a lower Pheophytin A peak) and the data indicate that several species of triacylglycerol are increased in the mutant (other peaks).

## V. Personnel Supported

This YIP grant supported Ru Zhang, a postdoctoral fellow who developed the ChlaMmeSeq tool and applied it to the screen for photosynthesis mutants.

Some limited support was also provided to postdoctoral fellows Mia Terashima, who developed the lipid screening tool, and to Xiaobo Li, who pursued the in-depth characterization of some of the hits from the lipid screen screen.

## VI. Publications

The ChlaMmeSeq tool and the findings it has enabled are published in the top primary research journal in plant biology, *The Plant Cell*:

High-Throughput Genotyping of Green Algal Mutants Reveals Random Distribution of Mutagenic Insertion Sites and Endonucleolytic Cleavage of Transforming DNA.

\*Zhang R, \*Patena W, Armbruster U, Gang SS, Blum SR, Jonikas MC.

\*Equal contribution

*Plant Cell*. 2014 Apr 7. [Epub ahead of print]

The pooled growth rate measurement tool and photosynthesis screen are currently being prepared for publication.

A manuscript describing our lipid screening technology has been reviewed favorably at a top journal in plant biology, and we are in the late stages of revising it for publication.

## VIIa. Interactions/Transitions: Presentations at meetings

Work resulting from this YIP grant was presented as three talks by the leading postdoctoral fellow on the YIP grant, Ru Zhang:

2013 - 22nd Western Photosynthesis Conference: "High Throughput Genotyping of Green Algal Mutants"

2012 - 15th Conference on the Cell & Molecular Biology of Chlamydomonas: "A novel, high through put genomic tool to comprehensively identify genes essential for photosynthesis and growth under high light in Chlamydomonas"

2012 - 21st Western Photosynthesis Conference: "Comprehensive identification of genes essential for photosynthesis and growth under high light using a novel, high throughput genomic tool"

Work resulting from this YIP grant was presented by the PI, Martin Jonikas, at the following conferences:

2014 - Gordon Research Conference on Photosynthesis Invited Talk: "Insights from efforts to engineer enhanced photosynthesis"

2014 - Bay Area Lipid Droplet Meeting: "High-throughput genetics of photosynthetic energy capture and storage"

2014 - UC San Diego Food & Fuel for the 21st Century Symposium Invited Speaker: "A new dawn for photosynthesis research: High-throughput genetics in algae"

2013 - Arizona State University Invited seminar: "High-throughput genetics of photosynthesis in algae"

2013 - 16th International Congress on Photosynthesis - "Genome-wide genotyping and phenotyping tools to transform our understanding of photosynthesis"

2013 - UC Davis Invited seminar: "Transforming our understanding of photosynthesis with high-throughput genetics in algae."

2012 - 15th International Conference on the Cell & Molecular Biology of Chlamydomonas, Potsdam, Germany: "Genome-wide mutagenesis and phenotyping tools to transform our understanding of photosynthesis"

2012 - Michigan State University student invited speaker: "Changing the pace of photosynthesis research by doing 100,000 experiments at once"

2011 - Alliance of Independent Plant Institutes invited talk: "Comprehensive identification of genes with roles in photosynthesis"

## **VIIb. Interactions/Transitions: Consultative functions**

Work funded by this YIP grant has not yet led to any consultative or advisory functions.

## **VIIc. Interactions/Transitions: Technology Assists**

Work funded by this YIP grant has not yet led to any technology assists, transitions or transfers.

## **VIII. New discoveries, inventions, or patent disclosures**

Work funded by this YIP grant has not led to any inventions or patent disclosures.

Briefly, the major discoveries that resulted from this YIP grant are as follows:

- Mutagenic insertions in Chlamydomonas are randomly distributed throughout the genome; thus it should be possible to efficiently obtain mutants in nearly all genes.
- During mutagenesis, insertion cassettes are truncated by the activity of a sequence-specific endonuclease. Removal of such sites from expression cassettes should facilitate expression of foreign genes in Chlamydomonas.
- During mutagenesis, fragments of genomic DNA from lysed cells appear to be ligated in between the transforming cassettes and the flanking genomic DNA. This leads to misleading flanking sequences.
- Many unexpected genes appear to be important for photosynthesis.
- There is a biological correlation between chlorophyll and lipid content among the cells of most strains, even when grown under a single condition. Each cell's lipid content as read out by Nile Red stain can be normalized by its chlorophyll content to yield improved separation between high-lipid mutants and wild-type on a flow cytometer; improving the accuracy of cell sorting.

## **IX. Honors/Awards**

We have been invited to speak about this work at 9 different meetings or seminar series.